AD_____

Award Number: DAMD17-98-1-8033

TITLE: Regulation of Breast Carcinoma Chemotaxis By The Integrin Alpha6Beta4

PRINCIPAL INVESTIGATOR: Kathleen L. O'Connor, Ph.D.
Arthur M. Mercurio, Ph.D.

CONTRACTING ORGANIZATION: Beth Israel Deaconess Medical Center
Boston, Massachusetts 02115

REPORT DATE: April 2001

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	April 2001	Annual Summary (16 Mar 00 - 15 Mar 01)

4. TITLE AND SUBTITLE

Regulation of Breast Carcinoma Chemotaxis By The Integrin Alpha6Beta4

5. FUNDING NUMBERS

DAMD17-98-1-8033

6. AUTHOR(S)

Kathleen L. O'Connor, Ph.D.
Arthur M. Mercurio, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Beth Israel Deaconess Medical Center,
Boston, Massachusetts 02115

E-Mail: koconnor@caregroup.harvard.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

Breast carcinoma invasion is a complex process in which the normal balance of cellular adhesion, proteolysis and directed migration is altered leading to penetration of the basement membrane and underlying stroma. Work from our lab has shown that expression of the integrin α6β4 in breast carcinoma cells enhances their invasiveness. With funding from this grant, I have shown that integrin α6β4 expression in breast carcinoma cells leads to an increase in chemotactic (directed) migration toward lysophosphatidic acid (LPA) and is required for the lamellae formation. Both lamellae formation and chemotactic migration are inhibited or 'gated' by cAMP. My results reveal that a critical function of α6β4 is to suppress the intracellular cAMP concentration by increasing the activity of a rolipram-sensitive, cAMP-specific phosphodiesterase (PDE). Also, my results show that the small GTPase RhoA controls lamellipodial formation, is required for directed migration and is preferentially activated by the α6β4 integrin. Furthermore, the ability of α6β4 to influence cAMP metabolism is critical to Rho activation and function. I also have found that cAMP metabolism and the α6β4 integrin can control the activation of Rac1, a protein that can counteract the functions of RhoA. The goal of this study is to understand how integrin α6β4 enhances lamellipodial formation and chemotaxis in breast carcinoma cells by identifying the signaling pathways involved in these processes. Toward this goal, I have made considerable progress and have created a firm basis for future work as an independent investigator.

14. SUBJECT TERMS

15. NUMBER OF PAGES
12

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction:

Tumor cell invasion and subsequent metastasis pose a serious threat to the survival of breast cancer patients. We currently need a better understanding of invasion in order to manage and eventually treat the late stages of breast cancer. The process of invasion requires a delicate balance between cellular adhesion, proteolysis and directed migration. The integrin α6β4, a receptor for laminin-1 (Lee, et al., 1992), laminin-2, laminin-4 (Spinardi, et al., 1995), and laminin-5 (Niessen, et al., 1994), has been linked to acquisition of an invasive phenotype and progression in multiple cancers (Falcioni, et al., 1994; Rabinovitz and Mercurio, 1996; VanWaes, et al., 1991). With breast cancer, recent studies have formed a strong link between integrin α6β4 and breast carcinoma invasion. First, Marchisio et al. have observed a striking localization of integrin α6β4 in invasive breast carcinoma. These observations support a previous study finding that expression of the integrin α6 subunit is more strongly associated with reduced patient survival than other markers including the estrogen receptor (Friedrichs, et al., 1995). Therefore, I believe that the integrin α6β4 plays a critical role in breast carcinoma invasion and progression and that the mechanism involved needs to be investigated.

Using an *in vitro* invasion assay system (Albini, et al., 1987), our group has shown that integrin α6β4, can enhance the invasive potential of MDA-MB-435 breast carcinoma (Shaw, et al., 1997) and the RKO colon carcinoma (Chao, et al., 1996) cell lines. Both the MDA-MB-435 and RKO cells express the laminin receptor integrin α6β1. Transfection of the integrin β4 subunit in these carcinoma cells results in the surface expression of the integrin α6β4 and increases the invasiveness of these cells. Furthermore, the expression of the integrin α6β4 does not lead to increased haptotactic migration on laminin for either the breast or colon carcinoma cell line. Since tumor cell invasion requires a delicate balance between cellular adhesion, proteolysis and directed migration, the integrin α6β4 signaling in this process likely involves the stimulation of one of these processes. I have recently resolved this issue by discovering that expression of the integrin α6β4 dramatically stimulates breast carcinoma cell chemotaxis, the directed migration toward a soluble attractant gradient, but not the proteolytic activity of these cells.

Currently, little is known about how integrin signaling impacts carcinoma cell chemotaxis and tumor progression. *Ultimately, the results from this study should help to delineate the role of α6β4 integrin in facilitating chemotaxis and shed light on general signaling mechanisms that lead to a more invasive phenotype that characterizes late stage breast cancer.* Toward this goal, I have made considerable progress during the three years funded by this fellowship.

Body:

The goal of this project is to identify signaling pathways involved in the invasion process that are enhanced by the α6β4 integrin. The α6β4 integrin was previously shown to promote carcinoma invasion by its activation of a phosphoinositide 3-OH (PI3-K) signaling pathway (Shaw, et al., 1997). As described in my 1999 annual report, I demonstrate using MDA-MB-435 breast carcinoma cells that α6β4 stimulates chemotactic migration, a key component of invasion, but that it has no influence on haptotaxis. Stimulation of chemotaxis by α6β4 expression was observed in response to either lysophosphatidic acid (LPA) or fibroblast conditioned medium. Moreover, the LPA-dependent formation of lamellae in these cells is dependent upon α6β4 expression. Both lamellae formation and chemotactic migration are inhibited or 'gated' by cAMP and our results reveal that a critical function of α6β4 is to suppress the intracellular cAMP concentration by increasing the activity of a rolipram-sensitive, cAMP-specific phosphodiesterase (PDE). This PDE activity is essential for lamellae formation, chemotactic migration and invasion based on data obtained with PDE inhibitors. Although PI3-K and cAMP-specific PDE activities are both required for promoting lamellae formation and chemotactic migration, our data indicate that they are components of distinct signaling pathways. The essence of these findings is that α6β4 stimulates the chemotactic migration of carcinoma cells through its ability to influence key signaling events that underlie this critical component of carcinoma invasion. **These data were published in the Journal of Cell Biology.**

Of particular relevance to my work, Butcher and colleagues reported that cAMP is a negative regulator of leukocyte migration signaled through the classical chemoattractants (Laudanna, et al.,

1997). In this model, cAMP impedes or gates RhoA-mediated leukocyte integrin activation and adhesion. Since LPA is a potent activator of RhoA (Moolenaar, et al., 1997), I wanted to explore the possibility that the integrin α6β4 releases cAMP gating of LPA-mediated RhoA activation that may lead to increased chemotaxis and lamellae formation. The Rho family of small GTPases, which includes Rho, Rac and cdc42, control the organization and remodeling of the actin cytoskeleton which is required for migration. These proteins have been implicated in the formation of stress fibers, lamellipodia and filopodia, respectively (Hall, 1998). Although much of our knowledge on their function has been obtained from studies with fibroblasts, other activities for these Rho GTPases have been observed recently in cells of epithelial origin. For example, Rac and cdc42 are required to maintain apical-basal polarity in epithelia (Jou and Nelson, 1998). Rho, interestingly, has been implicated in membrane ruffling in epithelial cells (Fukata, et al., 1999; Nishiyama, et al., 1994), a process attributed to Rac in fibroblasts (Hall, 1998). These findings are of particular interest with respect to our understanding of epithelial cell migration. The migration and invasion of epithelial-derived carcinoma cells are important phenomena that require the involvement of Rho GTPases (Itoh, et al., 1999; Keely, et al., 1997; Shaw, et al., 1997; Yoshioka, et al., 1998). For these reasons, we examined the hypothesis that RhoA is essential for the formation of actin-rich cell protrusions, the migration of carcinoma cells and, more importantly, that the activity of RhoA is regulated by the α6β4 integrin. In addition, we assessed the involvement of cAMP metabolism in these events.

For our initial studies, I used Clone A carcinoma cells which develop fan-shaped lamellae and exhibit random migration when plated on laminin-1, processes that are dependent on both the α6β4 and β1 integrins. To examine the hypothesis that RhoA functions in α6β4-dependent lamellae formation, clone A cells were co-transfected with a GFP construct and either a dominant negative RhoA (N19RhoA) or a control vector. Subsequently, the cells were plated onto laminin-1 and examined by phase-contrast microscopy. Clone A cells that expressed the control vector developed large lamellae with ruffled edges. In contrast, cells that expressed N19RhoA developed only a few small, fragmented lamellae that were devoid of membrane ruffles. Quantitative analysis of these images revealed that expression of N19RhoA reduced lamellar area by 80% in comparison to cells that expressed the control vector. Additionally, expression of N19RhoA inhibited the migration of clone A cells on laminin-1 by 70%. In contrast, expression of N17Rac did not inhibit the migration of clone A cells, although it did inhibit the migration of 3T3 cells by 85%. **These data, and the data described in the next four paragraphs, were published in the Journal of Cell Biology.**

Our observation that RhoA functions in lamellae formation and the migration of clone A cells, in conjunction with our previous finding that these events require the engagement of the α6β4 integrin (Rabinovitz and Mercurio, 1997), indicated that α6β4 may mediate the activation of RhoA. To assess RhoA activation, I used the Rho-binding domain of Rhotekin (RBD) to capture GTP-bound RhoA from cell extracts (Ren, et al., 1999). I find that the interaction of clone A cells with laminin-1, which requires α6β4, resulted in a significant amount of RhoA retained by RBD in comparison to the interaction of these cells with collagen I, which does not involve α6β4 directly. These experiments were performed with cells that had been attached to laminin for 30 minutes because membrane ruffling was most apparent at this time. Quantitative analysis of the results obtained in four independent experiments revealed a three-fold greater increase in RhoA activation in cells plated on laminin-1 than in cells plated on collagen. To establish the ability of α6β4 to activate RhoA more definitively, we used integrin-specific mAbs to cluster both α6β4 and β1 integrins. I find that clustering of α6β4 resulted in an approximate two to three-fold higher level of RhoA activity in comparison to cells maintained in suspension.

The involvement of cAMP metabolism in migration, lamellae formation and α6β4-mediated RhoA activation was investigated using both IBMX, a phosphodiesterase (PDE) inhibitor, and H-89, a cAMP-dependent protein kinase (PKA) inhibitor. IBMX treatment, which prevents cAMP breakdown, inhibited the migration of clone A cells on laminin-1 almost completely. In contrast, inhibition of PKA with H-89 increased the rate of migration by four-fold. Together, these data indicate that cAMP inhibits or 'gates' carcinoma migration and lamellae formation, in agreement with our previous findings

(O'Connor, et al., 1998). To establish the involvement of cAMP metabolism in the α6β4-mediated activation of RhoA, we used IBMX in the RBD assay. As shown in Fig. 3A, pretreatment of clone A cells with IBMX prior to plating on laminin-1 reduced the level of RhoA activation to that observed in cells plated on collagen. Importantly, IBMX did not inhibit either cell adhesion or spreading. Similar results were obtained with integrin clustering. These observations implicate cAMP metabolism in the α6β4-mediated activation of RhoA.

The data reported above raise the possibility that α6β4 influences RhoA localization because activation of RhoA is thought to involve its translocation to membranes (Bokoch, et al. 1994). To address this issue, clone A cells plated on either laminin-1 or collagen I were immunostained with a RhoA-specific Ab, as well as a β1-integrin specific Ab to mark membranes. In cells plated on collagen I, RhoA immunostaining was confined largely to the cytosol and it was distinct from the β1-integrin staining of the plasma membrane. In contrast, the α6β4-dependent interaction of clone A cells with laminin-1 resulted in the translocation of RhoA to membrane ruffles at the edges of lamellae where it co-localized with β1 integrin staining. However, RhoA did not co-localize with β1 integrins on the plasma membrane along the cell body. To assess the influence of cAMP metabolism on RhoA localization, clone A cells were pretreated with either IBMX or H-89 prior to plating on laminin-1. Inhibition of PDE activity with IBMX dramatically inhibited membrane ruffling and abolished RhoA localization in the few ruffles that persisted after IBMX treatment. Conversely, inhibition of cAMP-dependent PKA with H-89 resulted in an apparent increase in membrane ruffling and RhoA localization in membrane ruffles.

Together, these data contribute to our understanding of the mechanism by which α6β4 functions in the dynamic processes of cell migration and lamellae formation. I find that ligation of α6β4 with either antibody or laminin-1 results in the activation of RhoA, and its translocation from the cytosol to membrane ruffles at the leading edges of migrating carcinoma cells. Importantly, we also provide evidence that the α6β4-mediated activation of RhoA is necessary for lamellae formation, membrane ruffling and migration. Furthermore, we establish that these events are regulated by cAMP metabolism and that they can occur independently of Rac1 involvement.

During the last year of my fellowship, I have concentrated on the involvement of cAMP metabolism in the MDA-MB-435 breast carcinoma clones. In this system, the effects of cAMP metabolism is not as simple as in the clone A cells. We have previously shown that blocking cAMP phosphodiesterase activity with IBMX inhibits chemotaxis toward LPA in these cells (O'Connor, et al., 1998). Theoretically, blocking the downstream effector of cAMP, namely PKA, should reverse these effects. However this does not happen. As shown in Figure 1, the PKA inhibitor H-89 inhibited chemotaxis toward LPA in a dose-dependent manner. Furthermore, the effects of IBMX and H-89 were additive, suggesting that cAMP signaling and its downregulation through phosphodiesterases are both important for migration.

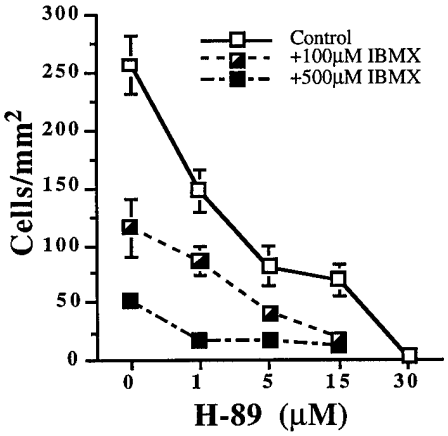

Figure 1. Chemotactic migration of MDA-MB-435 carcinoma cells requires both protein kinase A (PKA) and phosphodiesterase (PDE) activities. **(A)** MDA-MB-435 cells were treated with various concentrations of the PKA-specific inhibitor H-89 either alone (□) or in conjunction with 100μM (◪) or 500μM (■) of the PDE inhibitor IBMX. Cells ($5x10^4$) were then assayed for chemotaxis toward 100nM LPA for 4 hours in the continued presence of these inhibitors using a Transwell chamber assay. Values reported represent the mean number of cells migrated per mm^2 ± standard deviation obtained from triplicate samples.

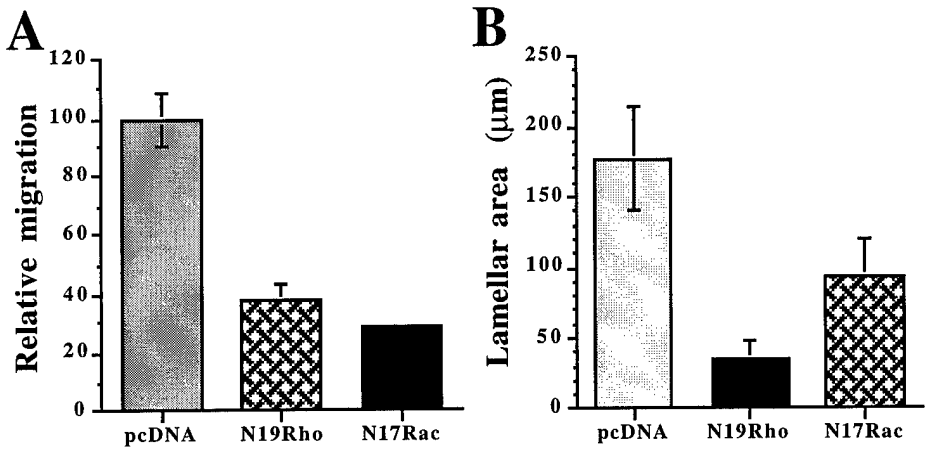

Figure 2. Dominant-negative constructs of RhoA or Rac inhibit chemotactic migration and lamellae formation in MDA/β4 cells. A) N19RhoA, N17Rac or vector constructs were co-transfected with β-gal cDNA into MDA-MB-435 cells that express Integrin α6β4 (MDA/β4). After 24 hours, cells were assayed for LPA-stimulated chemotaxis as described in Figure 1 and then stained for β-gal. Data are reported as the mean number of β-gal staining cells that have migrated versus negative control (vector alone) ± standard deviation of triplicate determinations. B) MDA/β4 cells were co-transfected with either vector, N19RhoA or N17Rac cDNA and a GFP reporter construct. After 48 hours, cells were plated onto LN-1 coated coverslips, treated with 100nM LPA for 5 minutes and then fixed. The percentage of GFP-positive cells that had lamellae was enumerated and reported from a representative experiment. Of note, N19RhoA and N17Rac expressing cells that formed lamellae had much smaller lamellae than control cells.

To address which Rho family GTPases were important for migration and lamellae formation in the MDA-MB-435 cells, I transiently transfected the MDA-MB-435 clones with a dominant negative construct of RhoA (N19 RhoA), Rac (N17Rac) or an empty vector. These transfectants were then assayed for their ability to chemotax or form lamellae in response to LPA. As shown in Figure 2, expression of dominant negative RhoA and Rac in the MDA/β4 cells inhibited chemotactic migration as compared to a vector only control. Interestingly, both constructs were also able to inhibit LPA stimulated lamellae formation in the MDA/β4 cells (Figure 2B). These data suggest that both RhoA and Rac are essential for chemotactic migration and lamellae formation in these cells.

Given that both β1 integrins (O'Connor, et al., 1998) and PKA (Fig. 1) are required for chemotaxis, I tested the possibility that β1 integrin signaling stimulates PKA activation. To address this hypothesis, β1 integrins were clustered with a specific mAb for varying periods of time and cell extracts were assayed for PKA activity. As shown, antibody-mediated clustering of β1 integrins resulted in a rapid, three-fold activation of PKA (Fig. 3) that decayed to baseline levels by 30 min (data not shown). The specificity of this activation is indicated by the absence of PKA activation by MHC class 1 clustering (Fig. 3). These data identify β1 integrin signaling is a potential source of PKA activation required for chemotactic migration.

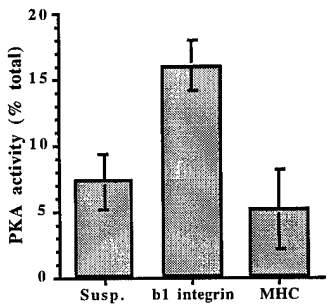

Figure 3. β1 integrin signaling activates PKA. MDA-MB-435 cells were maintained in suspension or incubated with either the β1 integrin mAb or an MHC-specific mAb and then added to anti-mouse IgG Ab-coated tissue culture plates. After 5 min., cells were extracted and assayed for PKA activity using a commercially available kit (Gibco-BRL). Values reported are the mean percent of total PKA activity ± standard deviation.

The small GTPase Rac is necessary for the migration and invasion of carcinoma cells (Keely, et al., 1997; Sander, et al., 1998) including MDA-MB-435 (Shaw et al., 1997). Based on the findings that PKA impedes Rho function and that Rac and Rho often differ in their function and regulation, we hypothesized that PKA activity is required for Rac activation. To test this hypothesis, we analyzed the activation of Rac by antibody-mediated clustering of β1 integrins. As shown in Fig. 4A, clustering of β1 integrins induced a time-dependent activation of Rac that was maximal at 30 min. Importantly, inhibition of PKA activity with either H-89 (Fig. 4B) or PKI (data not shown) prevented Rac activation by β1 clustering. These PKA inhibitors did not interfere with cell attachment to the Ab-coated wells (data not shown). Together, these results indicate that Rac activation in response to clustering of β1 integrins requires PKA activity. **These data are being organized into a manuscript for submission to the Journal of Biological Chemistry.**

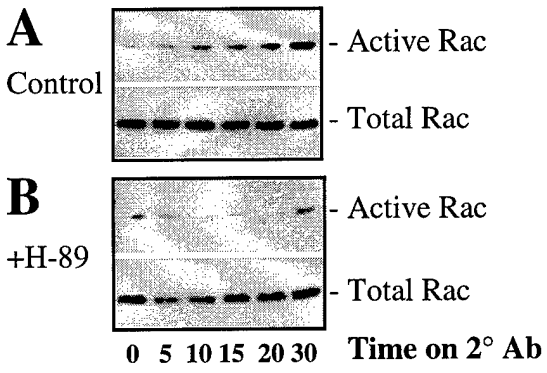

Figure 4. Activation of Rac1 by integrin clustering requires PKA. Antibody-mediated clustering of β1 integrins was performed as described in Fig. 2 either in the presence **(A)** or absence **(B)** of 15μM H-89 for times indicated. Cell extracts were assayed for Rac activation using Pak binding domain (PBD) assay as described (Sander, et al., 1998). Upper panels, Rac bound to the PBD (active Rac); Lower panels, Total Rac expressed in cell extracts.

Rac1 and RhoA are both required for LPA-stimulated chemotaxis and lamellae formation. I postulate that the integrin α6β4 promotes these events by enhancing the activation of these small GTPases due to its ability to enhance PI3K signaling and cAMP-specific PDE activity, respectively. For these experiments, I assayed either Rac (Sander et al., 1998) or Rho activity (Ren, et al., 1999) in MDA/β4 and MDA/mock cells was then assessed using the effector-binding affinity precipitation assays to capture GTP-bound proteins from cell extracts as described previously. As shown in Fig. 5, expression of integrin α6β4 promotes the activation of both Rac and Rho. Further studies have shown that this Rac activation is dependent on both PI3K and PKA activities, while Rho activation requires PDE activity (data not shown).

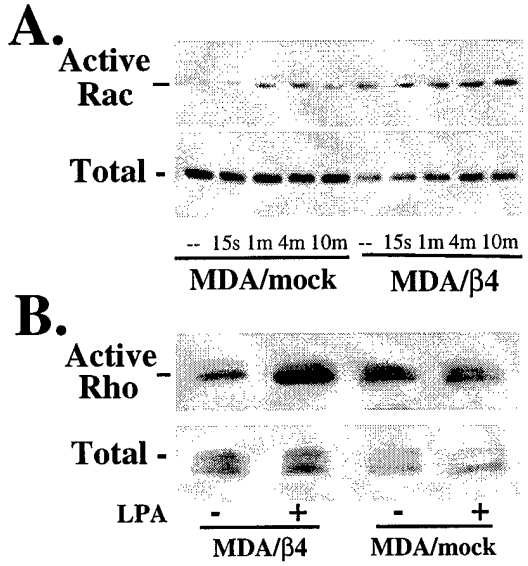

Figure 5. Activation of the small GTPases Rac and Rho by LPA is enhanced in integrin α6β4 expressing cells. (A) As noted, MDA-MB-435 clones were plated onto collagen-I coated dishes, allowed to attach and spread, treated with 100nM LPA and then assayed for Rac activity (Pak binding assay; Sander, et al, 1998) or Rho activity (Rhotekin-binding assay; Ren, et al., 2000). (A) Comparison of Rac activity in MDA/mock and MDA/β4 in response to LPA treatment for various time periods. (B) Comparison of Rho activity in the presence or absence of LPA (5 min.) treatment..

To distinguish responses that are altered by integrin α6β4 signaling, I investigated the potential of MDA-MB-435 transfectants to mobilize Ca^{++} in response to 10μM cyclopiazonic acid (CPA). For these experiments, single cells loaded with the Ca^{++} sensitive fluorescent dye, fura-2, following the method of Marks and Maxfield (Marks, et al., 1990) and assayed for calcium content using microspectrofluorometry. With the help of Dr. Hamid Akbarali, I find that the MDA/β4 cells had a lower intracellular Ca++ concentration ($[Ca^{++}]_i$) in response to CPA treatment than either the MDA/mock or MDA/β4ΔCYT clones. This increase in $[Ca^{++}]_i$ was sensitive to the concentration of extracellular Ca++ suggesting that this response is due to a capasitive Ca^{++} influx current (ICRAC; data not shown). This data suggests that expression of the *α6β4 integrin can alter mobilization of Ca^{++} by modulating I_{CRAC} channels.*

Addressing the Statement of works:

The research accomplishments of this fellowship have followed the approved statement of works with one possible exception. The Integrin α6β4 regulation of calcium mobilization study was not carried through to completion. This was the result of two major factors. First, the studies on the small GTPases was more labor intensive than originally thought. And secondly, my collaborator, Dr. Hamid Akbarali left BIDMC. The initiation of these studies, however, will allow for the basis of future studies that I hope to pursue once I establish my own research laboratory.

Key Research Accomplishments under this Fellowship:

- The integrin α6β4 stimulates the chemotactic migration of carcinoma cells. This ability to enhance directed migration is central to the ability of this integrin to promote invasion.
- Expression of the integrin α6β4 is required for lamellae formation in carcinoma cells in response to chemoattractants. Lamellae are actin rich structures associated with a motile phenotype.
- The integrin α6β4 increases the activity of a rolipram-sensitive, cAMP-specific phosphodiesterase (PDE4), which suppresses the intracellular concentration of cAMP. This PDE activity is required for carcinoma cell chemotaxis, invasion and lamellae formation.
- The integrin α6β4 promotes the activation of RhoA, a protein implicated in tumor progression and vital for remodeling the actin cytoskeleton.
- RhoA activation is dependent on PDE and is inhibited by PKA activity.
- Integrin α6β4 signaling promotes the recruitment of RhoA to membrane ruffles. This localization of RhoA is required for lamellae formation and is sensitive to cAMP metabolism.
- In addition to PDEs, chemotactic migration also requires Protein Kinase A (PKA) activity.
- PKA and PDEs work cooperatively, rather than antagonistically, to promote chemotactic migration. These findings suggest the possibility that localized fluctuations or gradients in $[cAMP]_i$ may be necessary for chemotactic migration.
- Carcinoma migration also requires engagement of β1 integrins. The clustering of β1 integrins leads to activation of PKA. This observation suggest that β1 integrins are the source of PKA needed for chemotactic migration of carcinoma cells.
- PKA activity is required for activation of Rac1. Rac1 is another Rho family GTPase that is important for tumor progression and the invasion of carcinoma cells.
- The Integrin α6β4 can enhance the activation of both Rho and Rac small GTPases.
- In summary, these findings suggest that cAMP signaling and the Rho family of GTPases are key signaling pathways in late stage carcinomas.

Reportable outcomes:

Final Reports: The following are a list of publications and meeting abstracts that have resulted from the research efforts supported by this fellowship:

1. O'Connor, K. L., L.M. Shaw, and A. M. Mercurio. 1998. Release of cAMP gating by the α6β4 integrin stimulates lamellae formation and the chemotactic migration of invasive carcinoma cells. *J. Cell Biol.* 143: 1749-1760.

2. O'Connor, K. L., L.M. Shaw, and A. M. Mercurio. 1998. Release of cAMP gating by the α6β4 integrin stimulates lamellae formation and the chemotactic migration of invasive carcinoma cells. *ASCB Annual Meeting Abstract.*
3. O'Connor, K. L. and A. M. Mercurio. 1998. Integrin α6β4 Promotes Lamellae Formation and the Chemotaxis of Invasive Carcinoma Cells by Releasing cAMP Gating of RhoA Activation. *The Function of Small GTPases: Keystone Symposium (meeting abstract)*
4. O'Connor, K. L. and A. M. Mercurio. 1999. RhoA Function in Lamellae Formation and Migration is Regulated by the α6β4 Integrin and cAMP Metabolism. *Joint Regulation of Signaling Pathways by Integrins and Growth Factors: Keystone Symposium (meeting abstract)*
5. O'Connor, K. L., Bao-Kim Nguyen, and A. M. Mercurio.2000. RhoA function in lamellae formation and migration is regulated by the α6β4 integrin and cAMP metabolism. J. Cell Biol. 148:253-258.
6. Mercurio, Arthur M., Robin E. Bachelder, Isaac Rabinovitz, Kathleen L. O'Connor, Taneli Tani and Leslie M. Shaw. 2001. The Metastatic Odyssey: The Integrin Connection. Surgical Oncology Clinics of North America. 10:313-328.
7. Arthur M.Mercurio, Robin E. Bachelder, Jun Chung, Kathleen L. O'Connor, Isaac Rabinovitz, Leslie M. Shaw and Taneli Tani. 2001. Integrin Laminin Receptors and Breast Carcinoma Progression. Journal of Mammary Gland Biology and Neoplasia. *In Press.*
8. O'Connor, K. L., L. M Shaw and A. M. Mercurio. 2000. Integrin α6β4 stimulates chemotactic migration of carcinoma cells by regulating cAMP metabolism. Department of Defense Era of Hope Breast Cancer Meeting, Atlanta, GA.
9. O'Connor, K. L. and A. M Mercurio. 2001. Cyclic AMP regulation of Rho family of GTPases. Keystone Symposium: Cell Migration and Invasion, Tahoe City, CA.
10. O'Connor, Kathleen L. and Arthur M Mercurio. Protein Kinase A Regulates Rac and is required for the Chemotactic Migration of Carcinoma Cells. In review.
11. O'Connor, Kathleen L. and Arthur M Mercurio. Integrin α6β4 control of the small GTPases Rac and Rho involved cAMP metabolism and PI3K. Manuscript in preparation.

There have been no patents or licenses, degrees, databases or animal models that have resulted from the funding of this fellowship. I have not applied for further funding.

Based on the work supported by this grant, I have applied for positions for assistant professor at several medical schools and research institutes around the country. These applications (approximately 50) have resulted in several interviews. I am currently in negotiations with the University of Texas Medical Branch in Galveston for a faculty position with the Sealy Cancer Center for Cell Biology and the Department of Surgery.

As a result of the funding from this award, I had the opportunity to spend two weeks with Marco Conti at Stanford University. Dr. Conti is a recognized expert in the area of cAMP phosphodiesterases. Although I did not generate any reportable data from this trip, I learned a great deal from Dr. Conti and his lab. Furthermore, I have established a collaboration with him that I hope to pursue in the coming years that will enable me to help further our understanding of carcinoma cell migration.

Conclusions:

During the three years of my fellowship, considerable progress has been made toward the understanding of the role of the α6β4 integrin in breast carcinoma invasion. The data that I have obtained demonstrate that the α6β4 integrin can stimulate lamellae formation and chemotactic migration of invasive carcinoma cells by increasing the activity of a rolipram-sensitive cAMP specific-PDE and lowering the $[cAMP]_i$. This cAMP specific-PDE functions in tandem with a PI3-K/Rac pathway, that is

also regulated by α6β4 (Shaw, et al. 1997), and is required for carcinoma invasion and lamellae formation. This work resulted in a publication in the Journal of Cell Biology (O'Connor, et al, 1998).

I have also obtained evidence that the target of cAMP-gating is RhoA, as has been observed previously in leukocytes. Using the Rhotekin binding assay to assess RhoA activation, we observed that engagement of α6β4 by either antibody-mediated clustering or laminin attachment resulted in a 2-3 fold increase in RhoA activation compared to cells maintained in suspension or plated on collagen. The α6β4-mediated interaction of clone A cells with laminin promoted the translocation of RhoA from the cytosol to membrane ruffles at the edges of lamellae and promoted its colocalization with β1 integrins, as assessed by immunofluorescence microscopy. In addition, RhoA translocation was blocked by inhibiting phosphodiesterase activity and enhanced by inhibiting the activity of cAMP-dependent protein kinase A. Together, these results establish a specific integrin-mediated pathway of RhoA activation that is regulated by cAMP and that functions in lamellae formation and migration. This work resulted in a publication in the Journal of Cell Biology (O'Connor, et al., 2000; publication appended to last year's report).

I have also found that the chemotactic migration of breast carcinoma cells toward either LPA involves not only PDE activity but also PKA-dependent signaling. Moreover, we demonstrate that the activation of the small GTPase Rac1 by either chemoattractant or β1 integrin clustering is regulated by PKA. Furthermore, we find that β1 integrin signaling stimulates the rapid and transient activation of PKA activity. A novel implication of these findings is that PKA can differentially regulate the activity and function of Rac and Rho and it may mediate a spatial and temporal regulation of these GTPases during migration. This work is being prepared for submission to the Journal of Biological Chemistry.

In summary, the data that I have obtained supports my hypothesis that the integrin α6β4 amplifies signals required for lamellipodial formation that helps to promote chemotaxis. This fellowship has permitted me to investigate key signaling events that underlie carcinoma invasion and, I hope, will advance our understanding and treatment of breast cancer.

References:

Albini, A., Y. Iwamoto, H. K. Kleinman, G. R. Martin, S. A. Aaronson, J. M. Kozlowski, and R. N. McEwan. 1987. A rapid *in vitro* assay for quantitating the invasive potential of tumor cells. *Cancer Res.* 47:3239-3245.

Bokoch, G. M., B. P. Bohl, and T.-H. Chuang. 1994. Guanine nucleotide exchange regulates membrane translocation of Rac/Rho GTP-binding proteins. *J. Biol. Chem.* 269:31674-31679.

Chao, C., M. M. Lotz, A. C. Clarke, and A. M. Mercurio. 1996. A function for the integrin α6β4 in the invasive properties of colorectal carcinoma cells. *Cancer Res.* 56:4811-4819.

Falcioni, R., V. Turchi, P. Vittulo, G. Navarra, F. Ficari, F. Cavaliere, A. Sacchi, and R. Mariani-Costantini. 1994. Integrin β4 expression in colorectal cancer. *Int. J. Oncology.* 5:573-578.

Friedrichs, K., P. Ruiz, F. Franke, I. Gille, H.-J. Terpe, and B. A. Imhof. 1995. High expression level of α6 integrin in human breast carcinoma is correlated with reduced survival. *Cancer Res.* 55:901-906.

Fukata, Y., N. Oshiro, N. Kinoshita, Y. Kawano, Y. Matsuoka, V. Bennett, Y. Matsuura, and K. Kaibuchi. 1999. Phosphorylation of adducin by Rho-kinase plays a crucial role in cell motility. *J. Cell Biol.* 145:347-361.

Hall, A. 1998. Rho GTPases and the actin cytoskeleton. *Science*. 279:509-514.

Itoh, K., K. Yoshioka, H. Akedo, M. Uehata, T. Ishizake, and S. Narumiya. 1999. An essential part for Rho-associated kinase in the transcellular invasion of tumor cells. *Nature Med.* 5:221-225.

Jou, T.-S., and W. J. Nelson. 1998. Effects of regulated expression of mutant RhoA and Rac1 small GTPases on the development of epithelial (MDCK) cell polarity. *J. Cell Biol.* 142:85-100.

Keely, J. P., J. K. Westwick, I. P. Whitehead, C. J. Der, and L. V. Parise. 1997. Cdc42 and Rac1 induce integrin-mediated cell motility and invasiveness through PI(3)K. *Nature*. 390:632-636.

Laudanna, C., J. M. Campbell, and E. C. Butcher. 1997. Elevation of intracellular cAMP inhibits RhoA activation and integrin-dependent leukocyte adhesion induced by chemoattractants. *J. Biol. Chem.* 272:24141-24144.

Lee, E. C., M. M. Lotz, G. D. Steele, and A. M. Mercurio. 1992. The integrin α6β4 is a laminin receptor. *J. Cell Biol.* 117:671-8.

Moolenaar, W. H., O. Kranenburg, F. R. Postma, and G. C. M. Zondag. 1997. Lysophosphatidic acid: G-protein signalling and cellular resposes. *Curr. Opin. Cell Biol.* 9:168-173.

Niessen, C. M., F. Hogervorst, L. H. Jaspars, A. A. deMelker, G. O. Delwel, E. H. Hulsman, I. Kuikman, and A. Sonnenberg. 1994. The α6β4 integrin is a receptor for both laminin and kalinin. *Exp. Cell Res.* 211:360-7.

Nishiyama, T., T. Sasaki, K. Takaishi, M. Kato, H. Yaku, K. Araki, Y. Matsuura, and Y. Takai. 1994. *rac* p21 is involved in insulin-induced membrane ruffling and *rho* p21 is involved in hepatocyte growth factor- and 12-0-tetradecanoylphorbol-13-acetate (TPA)-induced membrane ruffling in KB cells. *Mol. Cell. Biol.* 14:2247-2456.

O'Connor, K. L., B.-K. Nguyen, and A. M. Mercurio. 2000. RhoA Function in lamellae formation and migration is regulated by the α6β4 integrin and cAMP. *J. Cell Biol.* 148:253-258.

O'Connor, K. L., L. M. Shaw, and A. M. Mercurio. 1998. Release of cAMP gating by the α6β4 integrin stimulates lamellae formation and the chemotactic migration of invasive carcinoma cells. *J. Cell Biol.* 143:1749-1760.

Rabinovitz, I., and A. M. Mercurio. 1996. The Integrin α6β4 and the biology Of carcinoma. *Biochem. Cell Biol.* 74:811-821.

Rabinovitz, I., and A. M. Mercurio. 1997. The integrin α6β4 functions in carcinoma cell migration on laminin-1 by mediating the formation and stabilization of actin-containing motility structures. *J. Cell. Biol.* 139:1873-1884.

Ren, X.-D., W. B. Kiosses, and M. A. Schwartz. 1999. Regulation of small GTP-binding protein Rho by cell adhesion and the cytoskeleton. *EMBO J.* 18:578-585.

Sander, E. E., S. van Delft, J. P. ten Klooster, T. Reid, R. A. van der Kammen, F. Michiels, and J. G. Collard. 1998. Matrix-dependent Tiam1/rac signaling in epithelial cells promotes either cell-cell adhesion or cell migration is regulated by phosphatidylinositol 3-kinase. *J. Cell Biol.* 143:1385-1398.

Shaw, L. M., and A. M. Mercurio. 1994. Regulation of cellular interactions with laminin by integrin cytoplasmic domains: The A and B structural variants of the α6β1 integrin differentially modulate the adhesive strength, morphology, and migration of macrophages. *Mol. Biol. Cell.* 5:679-690.

Shaw, L. M., I. Rabinovitz, H. H.-F. Wang, A. Toker, and A. M. Mercurio. 1997. Activation of phosphoinositide 3-OH kinase by the α6β4 integrin promotes carcinoma invasion. *Cell.* 91:949-960.

Spinardi, L., S. Einheber, T. Cullen, T. A. Milner, and F. G. Giancotti. 1995. A recominant tail-less integrin β4 subunit disrupts hemidesmosomes but does not suppress α6β4-mediated cell adhesion to laminins. *J. Cell Biol.* 129:473-487.

VanWaes, C., K. F. Kozarsky, A. B. Warren, L. Kidd, D. Paugh, M. Liebert, and T. E. Carey. 1991. The A9 antigen associated with aggressive human squamous carcinoma is structurally and functionally similar to the newt defined integrin α6β4. *Cancer Res.* 51:2395-2402.

Yoshioka, K., F. Matsumura, H. Akedo, and K. Itoh. 1998. Small GTP-binding protein Rho stimulates the actomyosin system, leading to invasion of tumor cells. *J. Biol. Chem.* 273:5146-5154.